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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs) contribute to the majority of morbidity and mortality associated with NF1. The proposed studies will provide significant insight into one of the fundamental questions in neurofibroma biology: whether bi-allelic NF1 inactivation is necessary for neurofibroma formation. The objectives of this proposal are to use a newly established mouse model to (1) identify and characterize neurofibromas that are exclusively or predominantly comprised of NF1+/- cells (designated NF1+/- neurofibromas hereafter) in the skin and spinal roots; and (2) determine whether in this model, neurofibromas in the skin are similar to human dermal neurofibromas and thus are fundamentally different from the plexiform neurofibromas found in spinal roots. Previous studies of human tumors suggest that dermal and plexiform neurofibromas have fundamental differences in their dependence on the NF1 hetereozygous environment and have different malignant transformation potentials. We have made substantial progress in the first year of the award. For Task 1, we have generated most of the mutant mice proposed for the study. Phenotypic analysis of these mutant mice will be undertaken in the second year as proposed. For Task 2, we have completed most of the proposed experiments. We are writing a manuscript and trying to publish these results this year. For Task 3, we have generated the half of the mutant mice proposed for the study. The preliminary data suggest that the NF1 heterozygous environment is not essential for malignant transformation. This year, we will finishcharacterizing tumor phenotypes of these mutant mice and generate more mutants for analysis.

15. SUBJECT TERMS
neurofibroma, plexiform neurofibroma, mouse model, Schwann cell development, non-myelinating Schwann cells, myelinating Schwann cells, mast cell inflammation, axonal degeneration

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Introduction

Benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs) contribute to the majority of morbidity and mortality associated with NF1. The proposed studies will provide significant insight into one of the fundamental questions in neurofibroma biology: whether bi-allelic NF1 inactivation is necessary for neurofibroma formation. The objectives of this proposal are to use a newly established mouse model to (1) identify and characterize neurofibromas that are exclusively or predominantly comprised of $NF1^{+/-}$ cells (designated $NF1^{+/-}$ neurofibromas hereafter) in the skin and spinal roots; and (2) determine whether in this model, neurofibromas in the skin are similar to human dermal neurofibromas and thus are fundamentally different from the plexiform neurofibromas found in spinal roots. Previous studies of human tumors suggest that dermal and plexiform neurofibromas have fundamental differences in their dependence on the NF1 hetereozygous environment and have different malignant transformation potentials. **Thus, we will test three hypotheses: (1) bi-allelic NF1 inactivation is not necessary for neurofibroma formation; (2) An NF1 heterozygous microenvironment is not essential for neurofibroma formation in the skin; (3) neurofibromas in the skin and spinal roots have distinct tumorigenic potential in response to subsequent p53-mediated malignant transformation. To test these hypotheses, we propose the following specific aims.**

Body

Task 1. To determine whether NF1 heterozygous cells exclusively can give rise to neurofibromas in the skin and spinal roots.

We have established a genetic cross between $NF1^{flox/+}$;P0A-cre+ and $NF1^{flox/-}$;R26R-LacZ/R26R-LacZ mice to generate Schwann cell-specific mutant mice with the heterozygous and wild type background: $NF1^{flox/-}$;P0A-cre+;R26R-LacZ/+ (NF1 mutants with the NF1 heterozygous background) and $NF1^{flox/flox}$;P0A-cre+;R26R-LacZ/+ (NF1 mutants with the NF1 wild type background) along with the control littermates. We hereafter referred $NF1^{flox/-}$;P0A-cre+;R26R-LacZ/+ and $NF1^{flox/flox}$;P0A-cre+;R26R-LacZ/+ mice to as CKO1 and CKO2, respectively. Thus far, we have generated 16 CKO1 and 16 CKO2 mice, which are currently under monitor for neurofibroma formation. In addition, we have generated 3 CKO1 and 35 CKO2 mice without harboring the R26R reporter. We will isolate tumor tissues from these mice to establish molecular and biochemical assays proposed in this task.

Task 2. To examine whether an NF1 heterozygous microenvironment is essential for neurofibroma formation in the skin and spinal roots.

Peripheral nerves

Both CKO1 and CKO2 mice were viable, fertile and indistinguishable from their control littermates. However, in the second year of life, all the CKO1 mutant mice developed signs of sickness including lethargy, ruffled hair, skin lesions and hindlimb paralysis. Histological analysis revealed that all of the sick CKO1 mice (n = 14) exhibited tumor formation throughout the peripheral nervous system. We initially focused our analysis on sciatic nerves, because these nerves are the only parts of the peripheral nervous system in which the timing and stages of Schwann cell development are well-established. As compared to control nerves (Fig. 1A, E, I), all CKO1 sciatic nerves were significantly enlarged (Fig. 1B-D, J). Histological examinations of sciatic nerves revealed that 10 of 14 (71%) CKO1 mice developed full-blown neurofibromas (NF), which exhibited identical features of human counterparts composed of increased numbers of elongated spindle-shape cells and infiltrating mast cells in a matrix of rich collagen fibers (Fig. 1B, F and 1C, G). The neurofibroma tissues were always identified adjacent to hyperplastic lesions (Fig. 1B, C). The major pathological distinction between hyperplasia and neurofibroma is that hyperplasia does not disrupt normal nerve structure in spite of increased cellularity. Figure 1D shows an example of hyperplasia that exhibited dramatically increased cellularity with numerous blood vessels (arrowheads) and infiltrating mast cells (arrows). In contrast to the hyperplastic lesion shown in Fig. 1D, a neoplastic lesion identified in adjacent areas displayed a complete disruption of nerve structure evidenced by residual nerve fibers (arrows, Fig. 1H) and S100 staining (not shown). Compared to normal (Fig. 1E) and hyperplastic (Fig. 1D) nerves in which Schwann cells (SC) or Schwann cell-like cells align in parallel to nerve fibers, neurofibroma cells are typically distributed randomly in a collagen-rich matrix (Fig. 1F-H). The rest of the CKO1 mice without evidence of neurofibroma formation in sciatic nerves (n = 4, 29%) developed an intermediate lesion that we referred to as “hyperplasia with focal neurofibroma” (hyperplasia/NF). Hyperplasia/NF is characterized as an overall hyperplastic lesion with focal areas displaying neurofibroma features (Fig. 1N). Notably, the half of these 4 CKO1 mice also developed large cutaneous neurofibromas in the skin (see below), which may preclude hyperplasia/NF in sciatic nerves from progressing to full-blown neurofibromas.

Together, these observations indicate that NF1 inactivation in Schwann cell precursors is sufficient to initiate neurofibroma formation with high frequency.

To investigate the role of NF1 heterozygous environment in neurofibroma formation in this model, we analyzed 12 CKO2 mice at similar ages of the CKO1 mice described above. Most of the CKO1 and CKO2 mice analyzed were littermates (Fig. 1O). In contrast to CKO1 mice, none of the CKO2 mutant mice developed neurofibromas in sciatic nerves (Fig. 1K-N, 1O). Instead, three types of pre-neosplastic lesions were identified: hyperplasia (Fig. 1L, P), hyperplasia with focal demyelination (Fig. 1M, Q) (see below) and hyperplasia/NF (Fig. 1N, R). These results demonstrate that NF1 heterozygous environment is essential for neurofibroma formation in sciatic nerves.

Skin

In contrast to incomplete neurofibroma penetrance in sciatic nerves, all of the 20 CKO1 mice including 6 of those analyzed at early-stages developed plexiform subcutaneous neurofibromas. In the subcutaneous region of mouse skins, a thin skeletal muscle, known as cutaneus trunci muscle (CTM) or panniculus carnosus, covers very large portions of the trunk. As compared to control nerves (Fig. 2A, D, G), subcutaneous nerves underneath the CTM of CKO1 mice were greatly enlarged (Fig. 2B, 3L). Histological analysis revealed that these mutant nerves developed large plexiform neurofibromas (Fig. 2E, H), which were comprised of numerous Schwann cell-like cells completely unassociated or dissociating from axons, fibroblasts and mast cells (Fig. 2K, N), as compared to control nerves (Fig. 2J, M). For age-matched CKO2 mutant mice (Fig. 3I), approximately 42% of them developed pre-neoplastic lesions (Fig. 2C, F, I, L, O) similar to those observed in sciatic nerves. However, in contrast to sciatic nerves, about 33% of these CKO2 mice developed plexiform subcutaneous neurofibromas (Fig. 3A, D and 3B, E), which, in spite of similar morphology, were significantly smaller and contained fewer infiltrating mast cells as compared to CKO1 counterparts (Fig. 3G, J and 3H, K, L). One of the key differences between subcutaneous and sciatic nerves is that subcutaneous nerves are embedded in connective tissues with a large number of mast cells (Fig. 3D, E, J, K). Notably, the remaining 25% of the CKO2 mice analyzed exhibited a lesion that was rarely seen in CKO1 mutant nerves. These CKO2 nerves were characterized by peripheral disruption of the nerves (Fig. 3C) where only inflammatory cells were identified (Fig. 3F). These cells did not exhibit Schwann cell morphology or expressed Schwann cell markers, S100 or p75 (not shown). Along with low penetrance, the presence of such CKO2-specific lesions suggests that although the wild type environment in the skin can support tumor formation, but may not be completely permissive for neurofibroma cells.

Although all of the 20 CKO1 mice analyzed including those at early-stages developed subcutaneous neurofibromas, only one CKO1 mouse exhibited discrete cutaneous neurofibromas (Fig. 4A). Some of the cutaneous or subcutaneous neurofibromas observed in the CKO1 mice, but not in the age-matched CKO2 mice, infiltrated into the surrounding soft tissues, leading to hypertrophy of hindlimbs (Fig. 4B) and forelimbs (Fig. 4C). Figure 4D showed a trigeminal plexiform neurofibroma leading to overgrowth of facial soft tissues. Histological analysis revealed that neurofibroma cells infiltrated into normal skin tissues, blood vessels and adipose tissues (Fig. 4E, H). Similar to those observed in sciatic nerves, these cutaneous/subcutaneous neurofibromas expressed both S100 (Fig. 4F, I) and p75 (Fig. 4G, J). Importantly, these neurofibromas were morphologically indistinguishable from the lesions identified in the skin of human NF1 patients (Fig. 4K-P).

Task 3. To determine the malignant transformation potential of neurofibromas in the skin and spinal roots.

We have established the genetic cross between cisp53$^{+/-}$;NF1$^{+/flox}$;P0A-cre+ and NF1$^{flox/-}$;R26R-LacZ/R26R-LacZ mice to generate p53$^{+/-}$;NF1$^{-/flox}$;P0A-cre+;R26R-LacZ/+ (NF1 mutants with the NF1 and p53 heterozygous background) and p53$^{+/-}$;NF1$^{flox/flox}$;P0A-cre+;R26R-LacZ/+ (NF1 mutants with the NF1 wild type background and p53 heterozygous background) along with control mice. We hereafter referred p53$^{+/-}$;NF1$^{-/flox}$;P0A-cre+;R26R-LacZ/+ and p53$^{+/-}$;NF1$^{flox/flox}$;P0A-cre+;R26R-LacZ/+ mice to as p53CKO1 and p53CKO2, respectively. Thus far, we have generated 8 p53CKO1 and 8 p53CKO2 mice. Two of p53CKO1 and one of p53CKO2 already developed malignant peripheral nerve sheath tumors (MPNSTs). These preliminary observations suggest that the NF1 heterozygous environment is not essential for malignant transformation. Further analysis of these tumor-laden mice are under way.

Key research accomplishments

1. We have generated a first mouse model for neurofibroma in the skin.
2. We demonstrated that the NF1 heterozygous environment is essential for neurofibroma formation in the peripheral nerves, but not in the skin.
3. Our preliminary data suggest that the NF1 heterozygous environment is not essential for MPNST formation.

Reportable outcomes

1. We are preparing a manuscript, many of whose results were derived from our study of the Task 2.

2. Platform presentation, "Genetic analysis of peripheral nerve sheath tumor", 2006 Children's Tumor Foundation International Neurofibromatosis Consortium, Aspen, Colorado, June 4th – 6th, 2006.

3. We have generated a first mouse model for neurofibroma in the skin.

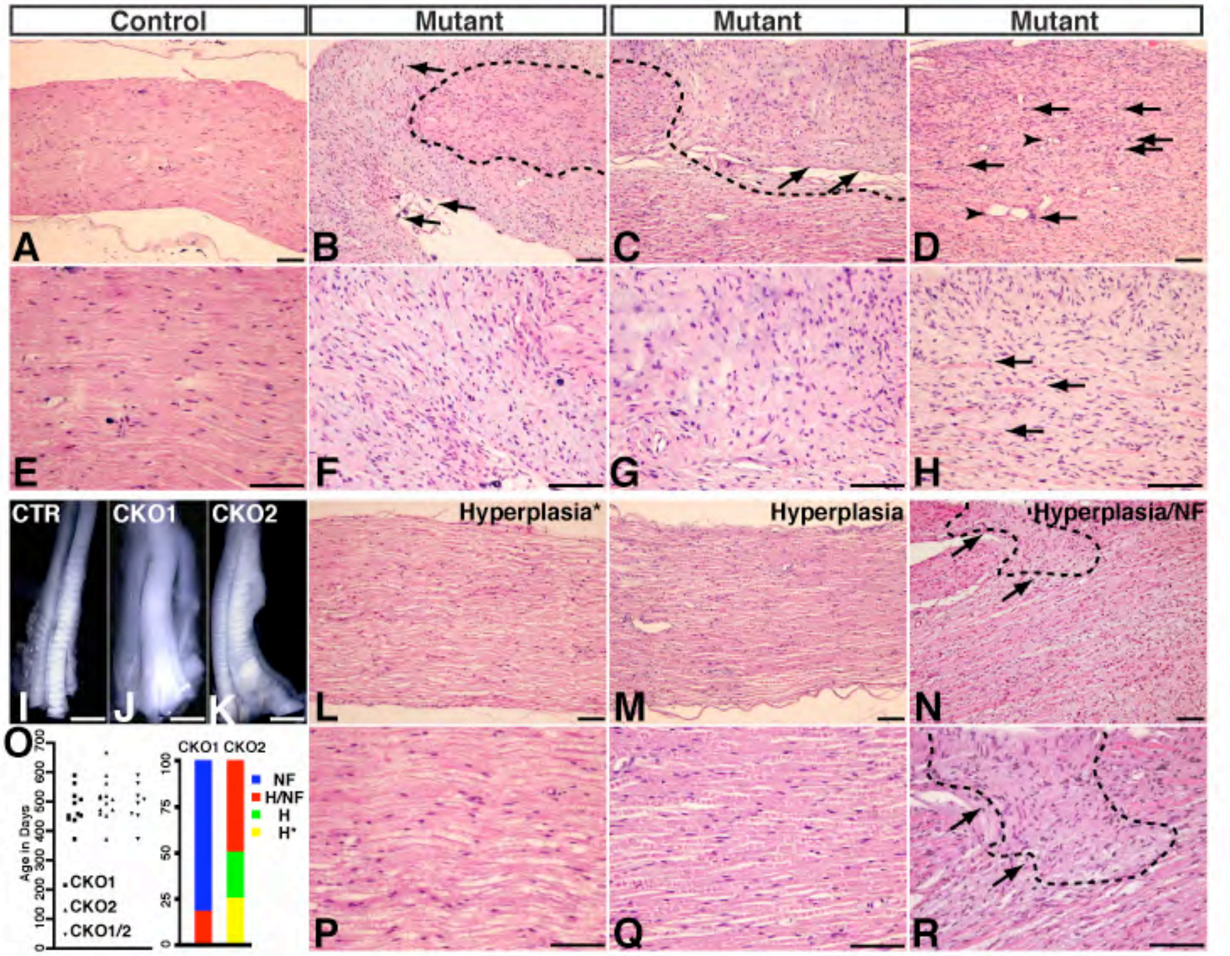
Conclusion

We have made substantial progress in the first year of the award. For Task 1, we have generated most of the mutant mice proposed for the study. Phenotypic analysis of these mutant mice will be undertaken in the second year as proposed. For Task 2, we have completed most of the proposed experiments. We are writing a manuscript and trying to publish these results this year. For Task 3, we have generated the half of the mutant mice proposed for the study. The preliminary data suggest that the NF1 heterozygous environment is not essential for malignant transformation. This year, we will finish characterizing tumor phenotypes of these mutant mice and generate more mutants for analysis.

Appendices and supporting data

Figures 1-4

Figure 1



Control
Mutant
Mutant
Mutant
A
B
C
D
E
F
G
H
CTR
CKO1
CKO2
I
J
K
Hyperplasia*
Hyperplasia
Hyperplasia/NF
L
M
N
O
Age in Days
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CKO2
CKO1/2
CKO1 CKO2
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H*
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Q
R

Figure 2

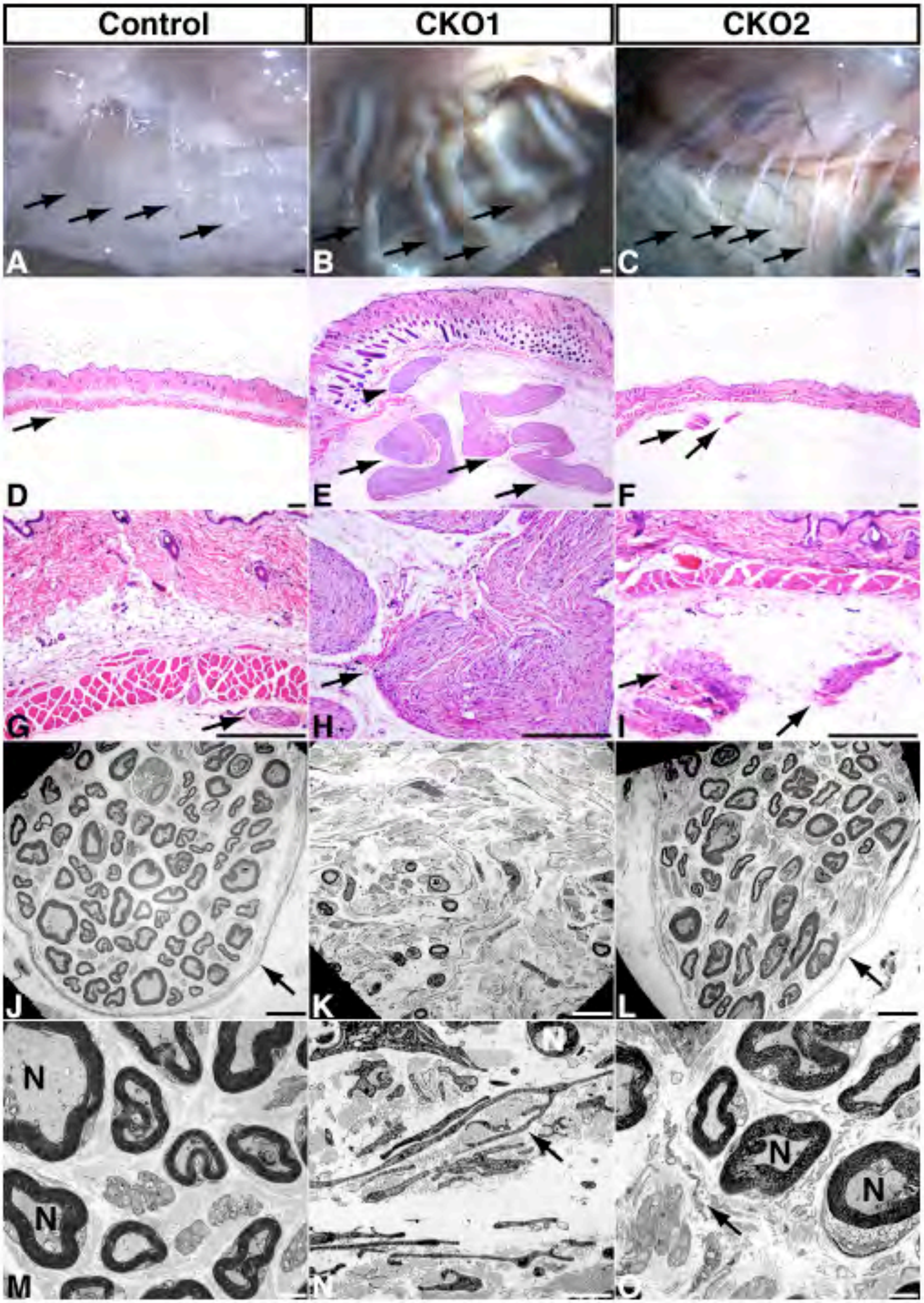

Figure 3

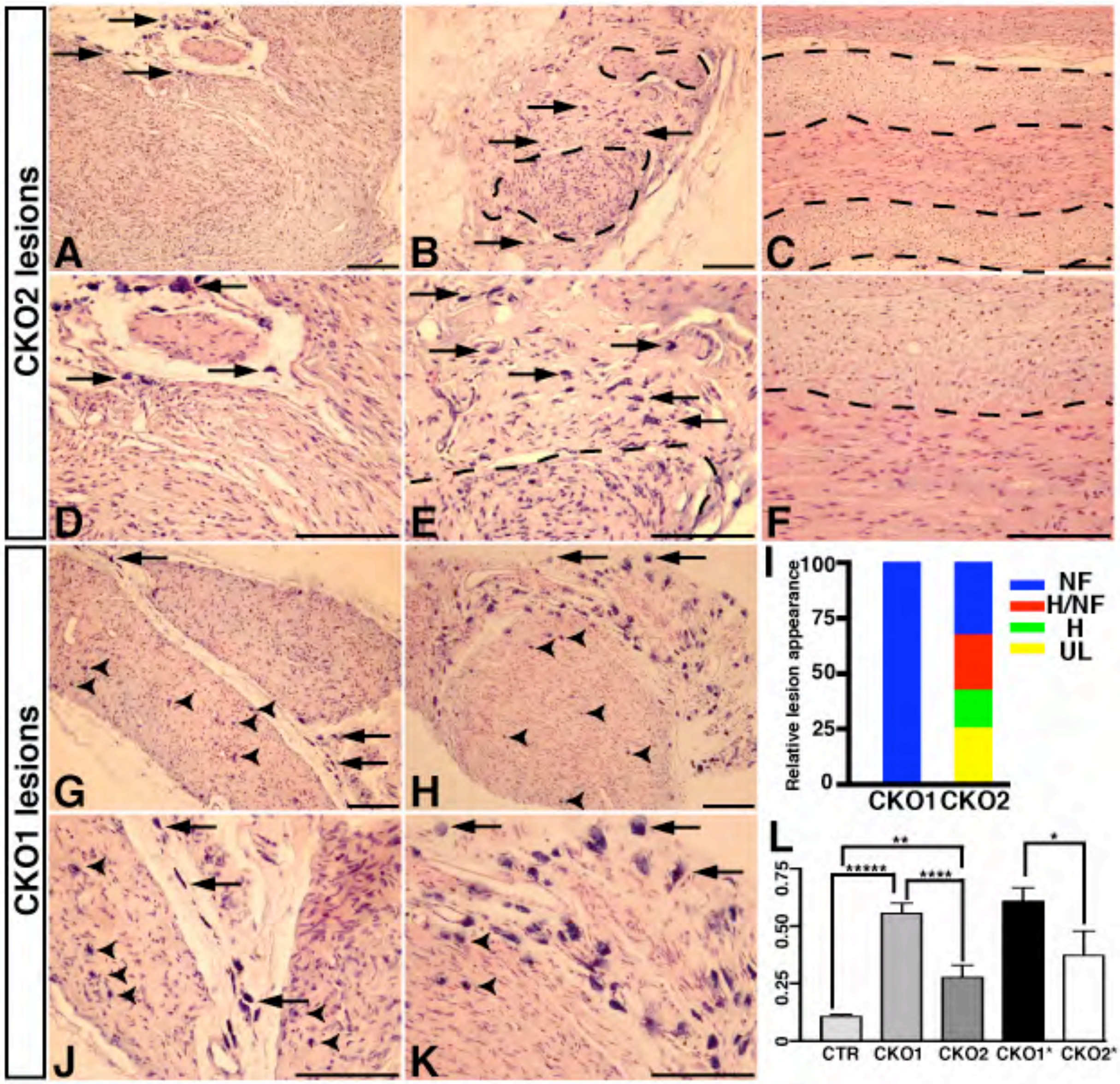
CKO2 lesions
CKO1 lesions
A
B
C
D
E
F
G
H
I
J
K
L
Relative lesion appearance
100
75
50
25
0
CKO1
CKO2
NF
H/NF
H
UL
0.75
0.50
0.25
0
CTR
CKO1
CKO2
CKO1*
CKO2*
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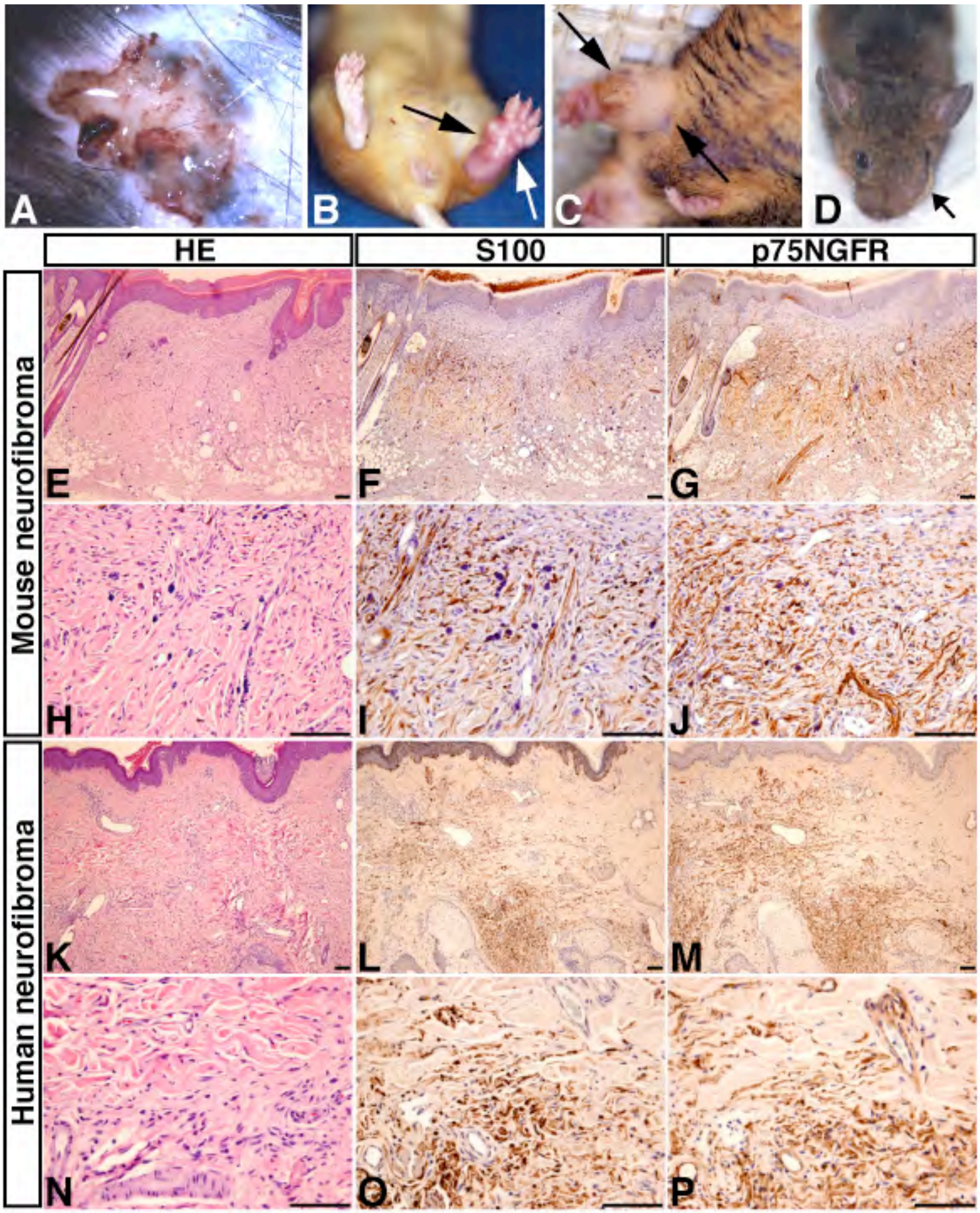
Figure 4


A
B
C
D
HE
S100
p75NGFR
Mouse neurofibroma
Human neurofibroma
E
F
G
H
I
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K
L
M
N
O
P